Internalization of Aeromonas hydrophila by fish epithelial cells can be inhibited with a tyrosine kinase inhibitor

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Aeromonas hydrophila is a Gram-negative bacterium that is pathogenic in fish, causing motile aeromonad septicaemia. It can enter (invade) fish cells, and survive as an intracellular parasite. The host-pathogen interaction and signal transduction pathway were studied by screening signal transduction inhibitors using carp epithelial cells and a virulent strain of the bacterium, PPD134/91. Genistein, a tyrosine kinase inhibitor, postponed internalization of A. hydrophila into host cells, suggesting that tyrosine phosphorylation plays a role in internalization. In contrast, staurosporine, a protein kinase C inhibitor, and sodium orthovanadate, a protein tyrosine phosphatase inhibitor, accelerated internalization of PPD134/91. Other virulent strains of A. hydrophila were also examined and it is likely that all strains, irrespective of serogroup, use the same signalling pathway to facilitate bacterial uptake.

Keywords: tyrosine kinase, Aeromonas hydrophila, fish epithelial cells

INTRODUCTION

Aeromonas hydrophila is the causative agent of motile aeromonad septicaemia, found in a wide variety of freshwater fish species (Newman, 1993; Thune et al., 1993). Motile aeromonad septicaemia outbreaks are common all over the world, and have been reported in Australia (Burke & Rogers, 1981), the south-eastern states of America (Pippy & Hare, 1969), Spain (Nieto et al., 1985) and the Southeast Asia region (Roberts et al., 1992). Outbreaks of motile aeromonad septicaemia usually occur only when the fish are immunocompromised by stresses such as overcrowding or concurrent disease (Stevenson, 1988). A. hydrophila produces several virulence determinants, including cytotoxins and enterotoxins (Ljungh & Wadstrom, 1982; Yadav et al., 1992) and a repertoire of enzymes that digest cellular components, mostly proteases and haemolysins (Allan & Stevenson, 1981; Leung & Stevenson, 1988). Other virulence factors such as the S layer (Dooley & Trust, 1988) and serum resistance (Mittal et al., 1980; Leung et al., 1995) are also implicated in aiding bacterial resistance to attack by the host's non-specific immune mechanisms.

A prerequisite for the initiation of infection is the initial adherence to, and invasion of, host epithelial cells. A. hydrophila is able to attach to collagen, fibronectin, serum proteins and glycoproteins found in fish mucus and epithelial cells (Atkinson & Trust, 1980; Ascencio et al., 1991; Neves et al., 1994). The putative adhesins thought to facilitate attachment may provide the necessary anchorage for the bacteria to facilitate subsequent invasion. We have recently studied the interaction between several different fish cell cultures and A. hydrophila and reported that A. hydrophila can enter into these cells and induce morphological changes (Leung et al., 1996; Low et al., 1998).

Invasion of cells by bacterial pathogens involves adherence of the organism to the host cell, followed by bacterial internalization into a membrane-bound vacuole inside the host cell (Moulder, 1985; Finlay & Falkow, 1989). Once inside the cell, these bacteria either remain within membrane-bound inclusions, as does Salmonella typhimurium, which multiplies efficiently within vacuoles (García-del Portillo et al., 1993), or escape into the cytoplasm, as does Shigella flexneri, which encodes enzymes to lyse the vacuolar membrane (Hale & Boventre, 1979).

Entry into non-phagocytic cells involves triggering host signal transduction mechanisms to induce rearrangements of the host cytoskeleton, thereby facilitating...
bacterial uptake (Finlay et al., 1991; Pace et al., 1993; Rosenshine & Finlay, 1993; Rosenshine et al., 1996). Initiation of cytoskeletal rearrangement by enteropathogenic *Escherichia coli* appears to be triggered by activation of specific host tyrosine kinase activity. The induction of tyrosine kinase activity is mediated by the products of the enteropathogenic *E. coli* cfm genes. Enteropathogenic *E. coli* also causes an increase in the level of intracellular free calcium (Rosenshine et al., 1992a). For *Yersinia* species, invasin interacts with host integrins, causing the clustering of integrin into focal points, which possibly triggers host tyrosine kinase activity that may result in host cytoskeletal rearrangements (Young et al., 1992). Thus, activation of tyrosine kinase appears to be a common feature in the signal transduction pathway of invasive pathogens leading to internalization.

The surface characteristics, virulence factors and distribution of *A. hydrophila* have been extensively studied. However, the interaction between the host and *A. hydrophila* remains largely unknown. In this work, we aimed to examine the interaction between *A. hydrophila* and carp epithelial cells. By using inhibitors of potential components of signal transduction pathways, it was hoped that the signalling pathway mediating internalization could be elucidated and thus that the pathogenesis of *A. hydrophila* would be better understood.

**METHODS**

**Bacterial strains.** Three virulent strains of *A. hydrophila* were used in this study (Leung et al., 1995). *A. hydrophila* PPD134/91, PPD122/91 and PPD11/90 were isolated from diseased fish by the Primary Production Department of Singapore. They were tested using standard biochemical diagnostic kits (Microbact 24E System, Medvet Science; and BBL Crystal Enteric/Nonfermenter ID System, Becton Dickinson), and their identities were confirmed according to the criteria of Popoff (1984). Cultures were routinely grown in tryptic soy broth (TSB; Difco) or on tryptic soy agar (TSA; Difco) at 25 °C. Stock cultures were maintained at −80 °C as a suspension in TSB containing 25% (v/v) glycerol.

**Cell cultures.** All tissue culture reagents were obtained from Gibco. EPC cells (epithelioma papillosum of carp, *Cyprinus carpio*) (Wolf & Mann, 1980) were grown in minimal essential medium (MEM) with Hanks' salts, 10 mM HEPES (pH 7:3), 2 mM glutamine, 0:23% NaHCO₃ and 10% heat-inactivated foetal bovine serum at 25 °C in a 5% CO₂ atmosphere. Cells were grown in 75 cm² flasks and split at least once a week by trypsin/EDTA treatment and dilution at 1:10 in fresh media.

**Confocal microscopy.** The Live/Dead BacLight Viability kit (Molecular Probes) was used to fluorescently label bacteria. NBD-phallacidin (Molecular Probes) was used to reveal the F-actin structure. EPC cells were seeded on glass coverslips and infected with *A. hydrophila* as described below. Coverslips were examined using a Nikon Optiphot microscope attached to the Bio-Rad MRC 500 confocal system (Lasersharp). Confocal images obtained under argon laser 488 nm blue excitation were photographed with a Polaroid Freeze-frame recorder using Kodak Tmax 100 film.

**Signal transduction inhibitors.** Inhibitors and their concentrations used are listed in Table 1. The solvent used to dissolve all chemicals was DMSO, except for sodium orthovanadate, which was dissolved in deionized water. Before use, the inhibitors were further diluted in MEM supplemented with 10% (v/v) foetal bovine serum and this was added to the cultured EPC cells. The concentrations of inhibitors used were according to the manufacturers’ recommendations. For gen-

<table>
<thead>
<tr>
<th>Class</th>
<th>Specific inhibitor</th>
<th>Concen used (µM)</th>
<th>Time taken for infected EPC cells to reach stage II morphology (min)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>58.3±2.9</td>
</tr>
<tr>
<td>Protein tyrosine kinase inhibitors</td>
<td>Genistein</td>
<td>50, 100, 150, 200, 250†</td>
<td>96.7±2.9†</td>
</tr>
<tr>
<td></td>
<td>Herbimycin A</td>
<td>10</td>
<td>58.3±3.0</td>
</tr>
<tr>
<td></td>
<td>Tyrophostin 47</td>
<td>50, 100†</td>
<td>60.7±4.9</td>
</tr>
<tr>
<td></td>
<td>Tyrophostin</td>
<td>50, 100</td>
<td>60.7±4.9</td>
</tr>
<tr>
<td>Genistein analogue</td>
<td>Daidzein</td>
<td>150</td>
<td>61.7±2.9</td>
</tr>
<tr>
<td>Protein tyrosine phosphatase inhibitor</td>
<td>Sodium</td>
<td>10</td>
<td>52.3±2.9†</td>
</tr>
<tr>
<td></td>
<td>orthovanadate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein kinase C inhibitors</td>
<td>Staurosporine</td>
<td>0.005</td>
<td>50.0±5.0†</td>
</tr>
<tr>
<td></td>
<td>Calphostin C</td>
<td>0.5</td>
<td>63.7±8.1</td>
</tr>
<tr>
<td>Protein phosphatase inhibitors</td>
<td>Okadaic acid</td>
<td>0.1</td>
<td>61.5±3.5</td>
</tr>
<tr>
<td></td>
<td>Calyculin A</td>
<td>0.0001, 0.001, 0.002†</td>
<td>57.0±1.4</td>
</tr>
<tr>
<td>Ca²⁺ channel blockers</td>
<td>Nifedipine</td>
<td>0.1, 1†</td>
<td>62.3±6.6</td>
</tr>
<tr>
<td></td>
<td>Verapamil HCl</td>
<td>1, 10†</td>
<td>58.7±4.0</td>
</tr>
</tbody>
</table>

*Values shown are the mean ± SD from duplicate wells in three separate experiments.
† Concentrations used for the effect on infected EPC cells.
‡ Those inhibitors where the time taken to reach stage II was significantly different from the control (P<0.05).
Effect of inhibitors on the morphological changes and viabilities in EPC and bacteria. Studies on morphological changes were conducted by seeding $3 \times 10^5$ fish cells into each well of a 24-well tissue culture plate (Falcon) and proceeding as described by Leung et al. (1996). Briefly, $5 \, \text{ml}$ stationary-phase cultures were prepared by inoculating TSB with $A. \text{hydrophila}$ from frozen glycerol stocks and incubating overnight at $25 \, ^\circ\text{C}$. Three hours before infection of cells, mid-exponential-phase cultures were prepared by diluting the bacteria $1:20$ in fresh TSB and incubating at $25 \, ^\circ\text{C}$ for $2.5 \, \text{h}$. Bacterial cells were pelleted and washed three times in PBS ($137 \, \text{mM} \, \text{NaCl}, 2.7 \, \text{mM} \, \text{KCl}, 4.3 \, \text{mM} \, \text{Na}_2\text{HPO}_4$ and $1.4 \, \text{mM} \, \text{KH}_2\text{PO}_4$ at pH 7.2) before adding $5 \, \mu\text{l}$ to each tissue culture well (approximately $5 \times 10^8$ bacteria). After inoculation, the 24-well tissue culture plate was centrifuged ($800 \, \text{g}$, $5 \, \text{min}$, $4 \, ^\circ\text{C}$), then incubated for $30 \, \text{min}$ at $25 \, ^\circ\text{C}$. Infected monolayers were washed once with MEM and then incubated in fresh, supplemented MEM. The morphology of EPC cells was examined every $5 \, \text{min}$ using an Axiosvert 25CFL phase-contrast inverted microscope (Carl Zeiss) at $\times 200$ magnification. In studies of different signal transduction inhibitors, EPC cells were incubated with the inhibitor for $30 \, \text{min}$ before bacterial infection. The time taken to reach each morphological stage was determined from duplicate wells in three separate experiments.

To investigate if the inhibitors could halt the morphological changes of previously infected EPC cells, the monolayers were first infected with bacteria for $5, 10, 15, 20, 30$ and $45 \, \text{min}$ before the addition of inhibitors. To investigate if the effects of the inhibitors on EPC cells were reversible, the monolayers were incubated with the inhibitors for $30 \, \text{min}$, and then washed twice with MEM to remove the inhibitors before infection with the bacteria.

To ensure that the inhibitors did not affect EPC cells, the cells were grown for $3 \, \text{h}$ in MEM containing genistein ($250 \, \mu\text{M}$), staurosporine ($0.005 \, \mu\text{M}$) or sodium orthovanadate ($10 \, \mu\text{M}$). A trypan blue exclusion assay was then used to determine whether treated EPC cells had similar viability to the untreated control. Similarly, $A. \text{hydrophila}$ was treated with each of these three inhibitors for $3 \, \text{h}$. Bacterial viability counts were determined before and after the $3 \, \text{h}$ incubation period. EPC and bacterial viabilities were determined by doing each experiment in triplicate.

Internalization assay. The invasion assay was as described by Leung & Finlay (1991) with minor modifications. Briefly, a monolayer of EPC cells was grown for $72 \, \text{h}$ in $24$-well tissue culture plates to $100 \, \%$ confluence. The cells were then washed with MEM and incubated with the respective inhibitors at $25 \, ^\circ\text{C}$ for $30 \, \text{min}$ before bacterial inoculation. The mixture was centrifuged for $5 \, \text{min}$ at $800 \, \text{g}$ at $4 \, ^\circ\text{C}$ and the plate was incubated for a further $30 \, \text{min}$ at $25 \, ^\circ\text{C}$. The wells were washed once with MEM to remove bacteria remaining in the medium and incubated for $30 \, \text{min}$ with MEM containing gentamicin ($100 \, \mu\text{g} \text{ml}^{-1}$) to kill any residual extracellular bacteria. The EPC cells were washed three times with MEM to remove any remaining gentamicin that could kill bacteria subsequently released from cells. Triton X-100 ($1 \, \text{ml}, 1 \, \%$) was added to each well to lyse the EPC cells and dilution series were plated on TSA to determine the number of viable intracellular bacteria. The invasion rate was calculated as a mean of three trials in triplicate TSA plates.

Statistical analysis. All results from morphological and invasion assays were expressed as means $\pm \text{SD}$. The data from these assays were analysed using one-way ANOVA followed by a Duncan multiple range test (SAS software). A value of $P<0.05$ was considered to be significant.

RESULTS

Morphological changes of EPC cells induced by $A. \text{hydrophila}$

Upon infection with the virulent $A. \text{hydrophila}$ strain PPD134/91, EPC cells underwent a series of morphological changes empirically described as stages I–III. An uninfected monolayer of EPC cells appeared as a smooth sheet with the cells adhering tightly to their neighbours (Fig. 1a). In stage I (43 min post-infection), the cells became slightly detached from one another (Fig. 1b). The smooth appearance was lost and the cells appeared darker. In stage II (58 min post-infection), the separation between cells became more apparent, large holes separated cells (about 50% of the cells remained attached to the tissue culture plate) and they were elongated to form long spindles (Fig. 1c). In stage III (about 90 min post-infection), the cells became rounded and the spindle connections were lost (Fig. 1d). Bacteria enclosed in a vacuole could be seen in the cells but no other cellular organelle was visible (Fig. 1g).

Effects of $A. \text{hydrophila}$ on F-actin distribution

The distribution of microfilaments (F-actin) was also examined during $A. \text{hydrophila}$ infection. In the control EPC cells, F-actin was arranged in a network and stress fibres were seen (Fig. 2a). Cells that were infected with PPD134/91 had localization of F-actin (actin clouds) (Fig. 2b). These actin clouds were observed in stages I and II but not in stage III of infected cells (data not shown).

Screening of signal transduction inhibitors

Different classes of inhibitors were screened to better understand the signal transduction pathway used by $A. \text{hydrophila}$ when entering EPC cells. The times taken to reach the respective stages were recorded for each inhibitor. Stage II was chosen as an end point in scoring because of the clarity of the morphological changes. Among all the inhibitors used for screening, only genistein, a tyrosine kinase inhibitor, delayed the time taken for induction of morphological changes in EPC cells (Fig. 1f), whereas staurosporine, a protein kinase C inhibitor, and sodium orthovanadate, a protein tyrosine phosphatase inhibitor, decreased the time taken for changes to occur (Table 1). Staurosporine accelerated the morphological changes of EPC cells to a greater extent than sodium orthovanadate (Table 1 and Fig. 1e). Inhibitors alone did not induce any morphological changes in control EPC cells.

Concentration effects of genistein

The effect of increasing concentrations of genistein on internalization of $A. \text{hydrophila}$ was examined by measuring the time taken for the EPC cells to reach stage...
Fig. 1. Phase-contrast and confocal micrographs of EPC cells infected with A. hydrophila PPD134/91. Different stages of infection and treatments are included. (a) Uninfected control; (b) stage I of infection (43 min); (c) stage II of infection (58 min); (d, g) stage III of infection (70 min); (e) 43 min with 0.005 μM staurosporine; and (f) 58 min with 250 μM genistein. In (g) A. hydrophila was stained using the Live/Dead BacLight Viability kit. Bacteria were seen inside the distorted and ghost-like EPC cells as indicated by the arrow in (g). Bar in (f) (also applies to a-e), 15 μm; bar in (g), 10 μm.
Signal transduction in *A. hydrophila* and fish cells

Fig. 2. F-actin-stained confocal micrographs of EPC cells infected with *A. hydrophila* PPD134/91. (a) Uninfected control; (b) stage I of infection. In the control EPC cells, F-actin was arranged in a network and stress fibres were seen. Actin clouds were visible during stage I of infection (marked by white arrow). Bar, 5 μm.

I and stage II morphology (Fig. 3) and by measuring the number of bacteria internalized (Fig. 4). With increasing concentrations of genistein, the time taken for EPC cells to reach stage II morphology was significantly increased \((P < 0.05)\) (Figs 1f and 4). Genistein (50 μM) had negligible effect on the morphological effects of PPD134/91 on EPC cells. However, with increasing concentrations of genistein from 100 μM to 250 μM, times taken to reach stages I and II increased significantly (Fig.

Fig. 3. Effect of different concentrations of genistein on morphological changes of EPC cells induced by *A. hydrophila* PPD134/91. Stage I (hatched bars) was defined as the time when the EPC cells became slightly detached from one another. The smooth appearance was lost and the cells appeared darker in colour. Stage II (open bars) was defined as the time when larger holes separate infected cells and about 50% of the cells remained on the tissue culture plate. The infected EPC cells were elongated to form long spindles. Values shown are the mean ±SD from duplicate wells in three separate experiments for each sample. Those concentrations where the time taken to reach stage II was significantly different from that in control cultures \((P < 0.05)\) are marked with asterisks.

Fig. 4. Effect of different concentrations of genistein on invasion rate in EPC cells. Values shown are the mean ±SD of three experiments in triplicate TSA plates for each sample. Those concentrations where the invasion rate was significantly different from that in control cultures \((P < 0.05)\) are marked with asterisks.

In the presence of 250 μM genistein, infected EPC cells took 30 min longer than the control cells to reach stage I morphology and 41 min longer to reach stage II morphology. The time interval between the start of
stage I and the start of stage II was not significantly affected by the increasing concentrations of genistein \((P > 0.05)\). The number of bacteria internalized in the presence of different concentrations of genistein was compared to an untreated control, which was taken to be 100%. Similar concentration-dependent inhibitory effects of genistein were observed in this invasion assay as in the morphological studies. The number of intracellular bacteria decreased with increasing concentrations of genistein. At 50 nM genistein there was no apparent effect on the number of internalized bacteria, whereas at 250 nM genistein the number of bacteria internalized were reduced to 23% of the control cultures (Fig. 4). For all subsequent experiments, morphological assays (time taken to reach stage II) were used to study the effects of inhibitors on the interaction between PPD134/91 and EPC cells as they were easier to perform and correlated well with the internalization assay.

The ability of genistein to delay the effects of PPD134/91 on EPC cells that were already infected with bacteria was investigated by adding different concentrations of genistein at 5, 10, 15, 20, 30 and 45 min after infection of cells (Table 2). It appeared that morphological changes could be delayed by genistein for the first 20 min after infection. If applied 20 min after infection, only the higher concentrations of genistein (250 and 200 nM) significantly \((P < 0.05)\) delayed morphological changes (Table 2). If applied 30 min after infection, even the highest concentration of genistein (250 nM) failed to delay the effects of PPD134/91 on EPC cells.

### Effects of inhibitors on the functions of PPD134/91 and EPC cells

The viability of *A. hydrophila* PPD134/91 treated with genistein (250 nM), staurosporine (0.005 nM) or sodium orthovanadate (10 nM) in MEM for 3 h was 96.9 ± 3.3%, 94.5 ± 6.4% or 104.1 ± 7.9% \((n = 3)\) of untreated controls, respectively. Hence genistein did not interfere with bacterial viability. The viability of EPC cells treated with genistein (250 nM), staurosporine (0.005 nM) or sodium orthovanadate (10 nM) in MEM for 3 h was 100% \((n = 3)\) of untreated controls. Thus, genistein does not interfere with the viability of EPC cells.

To determine whether genistein permanently reduced the ability of *A. hydrophila* to induce changes in EPC cells, the bacteria were incubated with 250 nM genistein for 30 min, washed and used to infect EPC cells. The time taken to reach stage II morphology (57 ± 2 min) was similar to that seen with the untreated bacteria (58 ± 3 min). Hence genistein did not impair the ability of *A. hydrophila* to induce morphological changes. To determine whether genistein permanently affected host cells, EPC cells were incubated with 250 nM genistein for 30 min. After the genistein was removed by washing, PPD134/91 induced stage II morphology in treated EPC cells at the same time (58 ± 1 min) as untreated controls (58 ± 3 min). Hence the effects of genistein on EPC cells are reversible. Similar results were found for staurosporine and sodium orthovanadate (data not shown).

### Effects of daidzein on internalization

Daidzein is an inactive analogue of genistein, and does not inhibit the activity of tyrosine kinase. To confirm that genistein specifically inhibited the action of tyrosine kinase, the effect of daidzein on the time taken to reach stage II was examined. It was found that daidzein had no significant effect on the morphological changes in EPC cells induced by *A. hydrophila* (Table 1).

### Effects of inhibitors on other virulent strains

The effects of the different signal transduction inhibitors on two other virulent strains of *A. hydrophila*, PPD122/91 and PPD11/90, were also examined. Genistein significantly delayed the time taken for induction of morphological changes in the EPC cells by either PPD122/91 or PPD11/90. In contrast, staurosporine

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**Table 2. Effect of addition of genistein at various time points after infection with *A. hydrophila* PPD134/91**

Values shown are the mean ± SD from duplicate wells in three separate experiments.

<table>
<thead>
<tr>
<th>Genistein concn (µM)</th>
<th>Time to reach stage II when genistein added after:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>0</td>
<td>58.3±2.9</td>
</tr>
<tr>
<td>50</td>
<td>58.3±2.9</td>
</tr>
<tr>
<td>100</td>
<td>70.0±5.0*</td>
</tr>
<tr>
<td>150</td>
<td>75.0±5.0*</td>
</tr>
<tr>
<td>200</td>
<td>80.0±5.0*</td>
</tr>
<tr>
<td>250</td>
<td>85.0±5.0*</td>
</tr>
</tbody>
</table>

* Those concentrations where the time taken to reach stage II was significantly different from that in control cultures \((P<0.05)\).
Table 3. Effects of inhibitors on morphological changes of EPC induced by other virulent strains of A. hydrophila

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Time taken to reach stage II (min)*</th>
<th>Strain PPD122/91</th>
<th>Strain PPD11/90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>93.3 ± 2.9</td>
<td>51.7 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Genistein, 50 μM</td>
<td>93.3 ± 2.9</td>
<td>51.7 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Genistein, 100 μM</td>
<td>103.3 ± 5.8†</td>
<td>61.7 ± 10.4†</td>
<td></td>
</tr>
<tr>
<td>Genistein, 150 μM</td>
<td>105.0 ± 5.0†</td>
<td>71.7 ± 2.9†</td>
<td></td>
</tr>
<tr>
<td>Genistein, 200 μM</td>
<td>106.7 ± 2.9†</td>
<td>76.7 ± 2.9†</td>
<td></td>
</tr>
<tr>
<td>Genistein, 250 μM</td>
<td>110.0†</td>
<td>83.8 ± 2.9†</td>
<td></td>
</tr>
<tr>
<td>Stauroporine, 5 nM</td>
<td>81.7 ± 2.9†</td>
<td>45.0†</td>
<td></td>
</tr>
</tbody>
</table>

*Values shown are the mean ± SD from duplicate wells in three separate experiments.
†Those inhibitors where the time taken to reach stage II was significantly different from the control (P < 0.05).

decreased the time taken for the changes to occur (Table 3).

**DISCUSSION**

Genistein was found to inhibit the rate of morphological changes induced by A. hydrophila, whereas staurosporine and sodium orthovanadate accelerated it (Table 1). Genistein is a specific inhibitor of tyrosine kinase, with negligible effect on the activity of serine and threonine kinases (Akiyama et al., 1987), whereas sodium orthovanadate is an inhibitor of protein tyrosine phosphatase (Leis & Kaplan, 1982). Because the effects of genistein and sodium orthovanadate are consistent with their antagonistic enzymic actions, it is likely that tyrosine kinase is involved in interactions between A. hydrophila and EPC cells. Stauroporine is known to inhibit protein kinase C in vitro and increases tyrosine phosphorylation in PC12 cells (Rasouly & Lazarovici, 1994). Therefore, the accelerated rate of morphological change produced by staurosporine might be associated with enhanced tyrosine phosphorylation. Verapamil and nifedipine are Ca²⁺ channel blockers (Varadi et al., 1995). Our data show that both verapamil and nifedipine failed to block the morphological changes induced by PPD134/91 in EPC cells, suggesting that the internalization process is not mediated by Ca²⁺ influx through voltage-dependent Ca²⁺ channels. Likewise, calphostin C, a potent protein kinase C inhibitor (Kobayashi et al., 1989), and okadaic acid and calyculin A, inhibitors of protein phosphatase (Ishihara et al., 1989), did not alter the morphological changes induced by PPD134/91, indicating that the protein kinase C pathway of signal transduction might not be involved in the bacterial internalization process.

On the basis of these results it appears that a tyrosine kinase is involved in the signalling pathway between A. hydrophila and the EPC cells. Genistein also inhibits bacterial invasion in Listeria monocytogenes (Tang et al., 1994) and enteropathogenic E. coli (Rosenshine et al., 1992b). Genistein, stauroporine and sodium orthovanadate could affect internalization by other non-selective mechanisms, including affecting bacterial viability or some bacterial function needed for internalization or the functions of EPC cells. Each of these possibilities was examined and the data show that these inhibitors did not affect the bacteria and the EPC cells in a non-specific manner. Thus, these inhibitors appear to act primarily by specifically affecting bacterial internalization.

Genistein was able to reduce morphological changes for up to 20 min after infection. Twenty minutes may be the time required for signal transduction initiated by tyrosine kinase to transmit to downstream signalling molecules and once this has been achieved the bacterial internalization process is irreversible (Table 2). The time taken from stage I to stage II was not significantly different (P > 0.05) with increasing concentrations of genistein (Fig. 3). This suggests that on reaching a stage I morphology (43 min for control cells), the signal generated by tyrosine kinase has already been transduced to a downstream level that cannot be blocked by a tyrosine kinase inhibitor, such as genistein.

Daidzein is an inactive analogue of genistein that does not inhibit the action of tyrosine kinase. We found that daidzein had no significant effect on the invasion rate of A. hydrophila on EPC cells. This further supports the hypothesis that genistein blocks A. hydrophila internalization specifically by tyrosine kinase inhibition.

**Signal transduction pathway of A. hydrophila**

Our results suggest that there is a signal transduction pathway participating in the interaction between the pathogen A. hydrophila and EPC cells. It is likely that A. hydrophila adheres to the host cell surface before internalization. The adhesins may be the O-antigen of lipopolysaccharide (Merino et al., 1996) or the outer-membrane protein isolated by Lee et al. (1997) which
mediate the adhesion and internalization of the bacterium. At present it is not clear whether *A. hydrophila* also produces invasin(s) to aid in internalization.

After adhesion, *A. hydrophila* probably initiates a signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signalling cascade that involves tyrosine kinase.

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**REFERENCES**


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